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Unexpected role of canonical aerobic methanotrophs in upland agricultural soils

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ABSTRACT

Aerobic oxidation of methane at (circum-)atmospheric concentrations (< 40 ppm_v) has long been assumed to be catalyzed by the as-yet-uncultured high-affinity methanotrophs in well-aerated, non-wetland (upland) soils, the only known biological methane sink globally. Although the low-affinity canonical methanotrophs with cultured representatives have been detected along with the high-affinity ones, their role as a methane sink in upland soils remains enigmatic. Here, we show that canonical methanotrophs can contribute to (circum-)atmospheric methane uptake in agricultural soils. We performed a stable-isotope $^{13}C-CH_4$ labelling incubation in the presence and absence of bio-based residues that were added to the soil to track the flow of methane. Residue amendment transiently stimulated methane uptake rate (< 50 days). Soil methane uptake was sustained throughout the incubation (130 days), concomitant to the enrichment of $^{13}C-CO_2$. The ^{13}C-enriched phospholipid fatty acids (PLFAs) were distinct in both soils, irrespective of amendments, and were unambiguously assigned almost exclusively to canonical alphaproteobacterial methanotrophs with cultured representatives. 16S rRNA and *pmoA* gene sequence analyses revealed that the as-yet-uncultured high-affinity methanotrophs were virtually absent in these soils. The stable-isotope labelling approach allowed to attribute soil methane uptake to canonical methanotrophs, whereas these were not expected to consume (circum-)atmospheric methane. Our findings thus revealed an overlooked reservoir of high-affinity methane-oxidizers represented by the canonical methanotrophs in agriculture-impacted upland soils. Given that upland agricultural soils have been thought to marginally or do not contribute to atmospheric methane consumption due to the vulnerability of the high-affinity methanotrophs, our findings suggest a thorough revisiting of the contribution of agricultural soils, and the role of agricultural management to mitigation of climate change.

1. Introduction

Increasing food supply to meet the global human population growth often results in the conversion of native to arable lands and intensification of agriculture. The change in land-use alters the soil physico-chemical parameters and nutrient turnover, in turn, induces a compositional shift in the soil methanotrophic community and abundance which severely impairs the methane sink function (Levine et al., 2011; Ho et al., 2015a; Tate et al., 2007, 2015; Malghani et al., 2016; Meyer et al., 2017). As methane is a potent greenhouse gas, accounting for up to 17% of global warming (IPCC, 2013), it is crucial to understand the sources and sinks of atmospheric methane in the ecosystem. Whether well-aerated, non-wetland (i.e., upland) agricultural soils are a weak sink or source of atmospheric methane (Ho et al., 2015a) after land-use conversion depends more strongly on changes in the methanotrophic population than on shifts among the methane-producing archaea (Meyer et al., 2017). (Circum-)atmospheric methane oxidation, defined here as oxidation of methane at < 40 ppm_v (Singh et al., 2016) (atmospheric methane at ~1.8 ppm_v), is thought to be catalyzed by the as-yet-uncultured high-affinity methanotrophs. These methanotrophs belong to a specialized group of methane-oxidizers that are taxonomically distinct from the low-affinity, canonical ones with

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cultured representatives (Holmes et al., 1999; Horz et al., 2002; Knief et al., 2003; Maxfield et al., 2008; Kolb, 2009; Levine et al., 2011). In contrast, genes regulating methane oxidation in a canonical methanotroph was only induced at > 600 ppm_v methane (Baani and Liesack, 2008). These methanotrophs act as a methane bio-filter at oxic-anoxic interfaces in methane-emitting environments (Ho et al., 2013). The high-affinity methanotrophs have resisted cultivation to date, and are defined based on their *pmoA* gene (structural gene for methane oxidation) sequences and PLFA profiles (Bull et al., 2000; Knief et al., 2003; Bodelier et al., 2009). Consequently, these biomarkers are used to detect the high-affinity methanotrophs in the environment.

High-affinity methanotrophs belong to *Gammaproteobacteria* (e.g., clades upland soil cluster γ: USC γ) and *Alphaproteobacteria* (e.g., clades USCα) (Knief et al., 2003; Horz et al., 2005; Kolb et al., 2005; Shrestha et al., 2012). Although there are no isolated representatives of high-affinity methanotrophs, the genomic potential of a candidate bacterium belonging to the USCα has recently been characterized using a targeted cell-sorting approach coupled to metagenomic analysis (Pratscher et al., 2018). Phylogenetic analyses of the 16S rRNA, as well as the *pmoA* gene placed this microorganism close to known cultured methanotrophs of the genus *Methylocapsa*. Additionally, a cultured alphaproteobacterial methanotroph (*Methylocystis*) has been proven to oxidize methane at atmospheric or low (< 600 ppm) methane concentrations (Baani and Liesack, 2008). *Methylocystis*, along with *Methylosinus* are the only known canonical methanotrophs harbouring an isozyme of the conventional pMMO (pMMO2), enabling methane oxidation at low concentrations (Yimga et al., 2003; Baani and Liesack, 2008). Despite the ability of these methanotrophs to oxidize methane at (circum-)atmospheric levels and the detection of other canonical methanotrophs in upland soils showing atmospheric methane consumption, it was long thought that the as-yet-uncultured high-affinity methane-oxidizers exclusively form the metabolically active population in native soils, as revealed by substrate labelling studies (Holmes et al., 1999; Knief et al., 2003; Kolb et al., 2005; Malghani et al., 2016; Pratscher et al., 2011, 2018). Hence, the role of the canonical methanotrophs as a methane sink in upland soils remains unclear.

In contrast to the canonical methanotrophs, the putative high-affinity methanotrophs are vulnerable to disturbances, requiring close to a century to recover in population and activity following abandonment of agriculture (Maxfield et al., 2008; Levine et al., 2011). Consequently, high-affinity methanotrophs are thought to be indigenous to native soils (i.e., relatively un-disturbed environments; forests, meadows, and grasslands) which were typically used as model ecosystems to investigate these microorganisms (Knief et al., 2003; see review Kolb, 2009). In a recent study, however, we showed that upland agricultural soils readily consume atmospheric methane, and the potential for methane oxidation was transiently, but significantly stimulated after the addition of bio-based residues (Ho et al., 2015a, 2017a); the elevated methane uptake was comparable to levels exhibited in native soils. The high-affinity methanotrophs were not detected in these soils despite atmospheric methane uptake and potential methane oxidation at circum-atmospheric levels (10–40 ppm) (Ho et al., 2015a). Therefore, we hypothesized that the agricultural soils harbor novel clades of high-affinity methanotrophs and/or we overlooked the potential role of canonical methanotrophs acting as a methane sink. This study aimed to determine the methane sink function in upland agricultural soils, and to resolve the active members contributing to (circum-)atmospheric methane oxidation in agriculture-impacted soils.

2. Materials and methods

2.1. Site description and mesocosm incubation

The mesocosm experiment was performed using sandy loam and clay soils, respectively collected from agricultural fields in Vredepeel (51°32′32“N, 05°50′54“E) and Lelystad (52°31′20“N, 05°34′57“E) belonging to the Wageningen University and Research, the Netherlands in August 2015. Detailed soil sampling procedure, subsequent processing (air-dried at room temperature and sieved to 2 mm), and soil physico-chemical properties were described before (Ho et al., 2015a, 2017a). These soils contained comparable organic matter content (LOI ~4.7%), while the sandy loam soil was more acidic (pH 5.4) than the clay soil (pH 7.6). The bio-based residues (i.e., sewage sludge and compost) were used in this study because of their stimulatory effect on atmospheric methane uptake (Ho et al., 2015a), and confirmed methane oxidation at 10–40 ppm after amendment in both soils. The sewage sludge (Vallei Veluwe, the Netherlands) and compost (Recomede, the Netherlands) had been characterized (Ho et al., 2015a). The sewage sludge contained a higher total carbon (322 μg C mg dw $sample^{-1}$) and nitrogen (59 μg N mg dw $sample^{-1}$) content than the compost (total C: 140 μg C mg dw $sample^{-1}$; total N: 9 μg N mg dw $sample^{-1}$). As before (Ho et al., 2015a), the residues were oven-dried at 30 °C, crushed, and sieved to 2 mm prior to use.

Mesocosms were set up in six replicates in pots with a working dimension of 22 cm × 10 cm (diameter x height) as described before (Ho et al., 2017a). Briefly, each mesocosm consisted of 2.5 kg dry weight (dw) soil collected from the upper 15 cm in the field amended with residues (dw) to achieve a rate of 20 ton ha^{-1} (soil density, 90–110 kg m^3) comparable to agricultural practice (Diacono and Montemurro, 2010). Mesocosms containing only soil (un-amended) served as a reference. Deionized water was added and soil moisture was maintained at 65% of the water retention capacity throughout the incubation. Incubation was performed at 15 °C in the dark in a climate-controlled chamber. Soil was sampled after 7 days using an auger (diameter x height: 3 cm x 10–12 cm). The soil was homogenized by hand, sieved (< 2 mm), and stored in the 4 °C fridge overnight prior to further incubation. The mesocosm incubation provided the starting material for subsequent microcosm incubation in the laboratory to determine the composition of the metabolically active methanotrophs *via* $^{13}C{-}CH_4$ labelling and identification of the ^{13}C-enriched PLFA.

2.2. Microcosm incubation, and headspace methane and carbon dioxide measurements

The microcosm incubation was performed in 260 ml opaque bottles (Sercon Group, Crewe, UK), and consisted of 7.5 g fresh weight soil sampled from the mesocosm. Incubation was performed in triplicate under an initial headspace methane (un-labelled-/$^{13}C{-}CH_4$; Campro Scientific, Veenendaal, the Netherlands) concentration of ~40 ppm_v in air at 15 °C. To ensure sufficient ^{13}C labelling, headspace air and methane (un-labelled-/$^{13}C{-}CH_4$) were replenished twice (at days 50 and 100) when methane concentration had decreased > 50% of the starting amount. To replenish the headspace gas, the bottle was flushed with synthetic air comprising of 20% O_2 and 80% N_2 prior to unlabeled-/$^{13}C{-}CH_4$ addition. Incubation was stopped after 130 days when ^{13}C-derived CO_2 ($^{13}C{-}CO_2$) was enriched. An aliquot of the soil (5 g) was stored at 4 °C for PLFA extraction, while the remaining soil was kept in the −20 °C freezer for DNA extraction and subsequent molecular analyses.

Headspace methane was monitored during the incubation using an Ultra GC gas chromatograph (Interscience, Breda, the Netherlands) equipped with a Flame Ionization Detector (FID). The methane uptake rate was determined by linear regression of the methane depletion curve. The ^{13}C- and $^{12}C{-}CO_2$ ratio ($\delta^{13}C{-}CO_2$, ‰) was determined using the Ultra GC gas chromatograph (Interscience, the Netherlands) equipped with a FID and Rt-QBOND (30 m, 0.32 mm, ID) capillary column. Helium was used as the carrier gas, and the oven temperature was kept at 50 °C with a flow of 5 ml. $^{13}C{-}CO_2$ was analysed using a ThermoScientific gas chromatograph with combustion interface (Conflo II) system connected to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen, Germany). Samples (250 μl) were injected into the split injector (split ratio 1:10), and

eluted with Helium (5 ml min^{-1}) on Rt-QBOND (30 m, 0.32 mm, ID) capillary column at 31 ^{O}C. The reference gas was calibrated with methane δC (VPDB) −38.25 (Arndt Schimmelmann, Indiana University, Bloomington, USA).

2.3. Phospholipid fatty acid (PLFA) extraction, and ^{13}C-enriched PLFA identification

PLFA was extracted from 4 g of soil following the procedure of Frostegård et al. (1993) and Hedlund (2002), based on the method of Bligh and Dyer (1959) and White et al. (1979). PLFA extraction was analysed on a gas chromatograph (GC-FID, 7890A, Agilent technologies, Delaware, USA) to determine the abundance of the PLFA biomarkers. Identification of FAMEs was based on comparing retention indices data generated by GC-FID/GC-mass spectrometry (GC-MS; Thermo Finnagan TRACE GC-MS system) analysis with known standards and previously analysed reference samples as described before (Bodelier et al., 2013; Henneberger et al., 2015). The δ^{13}C value for each PLFA biomarker was determined by analysing PLFA extractions on a Thermo Trace Ultra GC, interfaced with a Thermo Scientific Delta V IRMS. For both GC analyses, an Agilent HP-5MS UI column (60m, 0.25 mm id, 0.25 µm film thickness) was used. The δ^{13}C PLFA of ^{13}C-labelled and un-labelled control samples was used to calculate the actual excess amount of ^{13}C in each PLFA biomarker (Boschker, 2004).

2.4. DNA extraction, and pmoA-based quantitative PCR (qPCR) assay

DNA was extracted from soil using the PowerSoil®DNA Isolation Kit (MOBIO, the Netherlands) according to the manufacturer's instruction. DNA extraction was performed in triplicate per treatment, soil type, and time (start and end of microcosm incubation). DNA extract was stored at −20 °C till further molecular analyses.

The qPCR assay targeting the *pmoA* gene (A189f/Mmb661r primer combination) was performed using the Rotor-Gene Q real-time PCR cycler (Qiagen, Venlo, the Netherlands). Each qPCR reaction (total volume, 20 µl) comprised of 10 µl 2X SensiFAST (Bioline, Alphen aan den Rijn, the Netherlands), 3.5 µl of each primer (5 pmol μl^{-1}), 1 µl bovine serum albumin (5 mg ml^{-1}; Invitrogen, Breda, the Netherlands), and 2 µl of diluted (50- or 10-fold) DNA extract. Previously, using the same soil, DNA extracts diluted 50- and 10-fold, respectively for the sandy loam and clay soil yielded the maximum *pmoA* gene copy numbers (Ho et al., 2015a). The PCR thermal profile consisted of an initial denaturation step at 95 °C for 3 min, followed by 44 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 10 s, and elongation at 72 °C for 25 s; data acquisition was performed at 87 °C for 8 s. The melt curve was obtained from 70 °C to 99 °C at 1 °C increment. In addition to the melt curve, amplicon specificity was checked in 1% agarose gel electrophoresis. Plasmid DNA isolated from a pure culture (*Methylosinus trichosporium* OB3b) was used to generate the calibration curve.

The apparent cell-specific activity was determined considering the initial methane uptake rate derived from days 0–50, and at days 101–130 (end rate), and the qPCR analysis (start and end points) for each soil and amendment. We assumed that a methanotroph, on average, harbours two *pmoA* gene copies (Semrau et al., 1995).

2.5. 16S rRNA and pmoA gene amplicon sequencing and analysis

The 16S rRNA and *pmoA* genes were amplified from soil DNA extracts after the microcosm incubation (day 130) using the 341F/799R and A189f/A682r primer pairs, respectively. The 341F/799R primer pair (Muyzer et al., 1993; Chelius and Triplett, 2001) targets the V4 region of the bacterial 16S rRNA gene, and covers the major phyla of the domain Bacteria (Ho et al., 2017a). Amplification was performed with a two-step approach as described before (Herbold et al., 2015; Reumer et al., 2018). In the first PCR, target genes were amplified with diagnostic primers synthesized with a 16 bp head sequence 5′-GCTAT GCGCGAGCTGC-3'. In the second PCR, amplification was performed with primers that consist of the 16 bp head sequence and include at the 5′- end, a library-specific 8 bp barcode, later used to sort the different samples. The contents of the PCR reaction and thermal profile is given in the Supporting Materials.

The 16S rRNA and *pmoA* gene sequencing reads were assembled by 'make.contigs' command in the Mothur program v 1.35.1 (Schloss et al., 2009). The assembled contigs were then sorted based on their length and the quality of the primers (≤2 errors), spacer (≤2 errors), and barcodes (≤1 error); barcodes and primers were subsequently removed. The 16S rRNA gene sequences were processed following the Miseq standard operating procedure (SOP) pipeline (Kozich et al., 2013; https://www.mothur.org/wiki/MiSeq_SOP). The remaining reads were then pre-clustered to de-noise sequences within each sample, and chimeric sequences were removed using 'chimeric.uchime' command (Edgar et al., 2011). Taxonomic classification was performed for all high-quality sequences at the genus level based on the SILVA database (v.123) using the nearest neighbor joining method. The *pmoA* gene sequences were analysed as detailed in Ho et al. (2018). Putative chimeric reads were removed by 'chimera.uchime' command with 'self' option in the Mothur program v 1.35.1. The high quality *pmoA* gene sequences were classified by BLAST/LCA method using a curated *pmoA* gene database and MEGAN tree as previously described (Dumont et al., 2014). Principal Component Analysis (PCA) was performed using the 'prcomp' command, and visualized using the 'ggfortify' package in R (v 3.4.1) for the 16S rRNA gene sequences (R Core Team, 2014). The 16S rRNA and *pmoA* gene sequences were deposited at the EMBL European Nucleotide Archive (ENA) under the project accession number PRJEB27460.

2.6. Statistical analyses

The level of significance between the methane uptake rates and $^{13}C/^{12}C{-}CO_2$ values exhibited in the different treatments was respectively determined by pairwise comparison of the residue- and un-amended soils, and in the $^{13}C{-}CH_4$ and un-labelled incubations per time using Student's t-test in SigmaPlot version 13.0 (Systat Software, Inc., USA).

The ^{13}C-enriched PLFA profiles of incubated samples were compared to profiles of cultured methanotrophs (Table S1) using metric multidimensional scaling (MDS) based on Bray-curtis similarity as described before (Bodelier et al., 2013). The MDS was executed in the PAST software version 3.20 (Hammer et al., 2011).

3. Results

3.1. Response of the methane uptake rate to bio-based residue amendments

Methane uptake rate was significantly stimulated after bio-based residue amendment when compared to the un-amended incubation ($P < 0.05$), and the increased activity was sustained up to 50 days in the compost-amended soils (Fig. 1). The stimulatory effect of the residues on the soil methane uptake rate diminished over time. Potential methane oxidation was further confirmed by analysis of the $^{13}C/^{12}C$ isotope ratio of CO_2, showing significant accumulation of $^{13}C{-}CH_4$-derived CO_2 ($^{13}C{-}CO_2$) over time when compared to the un-labelled reference incubation (Fig. 2). Generally, the $^{13}C/^{12}C{-}CO_2$ values were appreciably higher in the clay soil, indicating enrichment of $^{13}C{-}CO_2$ which corroborated with the higher mean methane uptake rate (Figs. 1 and 2). $^{13}C{-}CO_2$ was highly enriched in the compost-amended soils when compared to the sewage sludge-amended soils after incubation. However, values in the compost-amended soils were comparable to their respective un-amended soils (Fig. 2). With proven methane oxidation capacity at circum-atmospheric methane levels and significant enrichment of $^{13}C{-}CO_2$, samples were collected for PLFA extraction.

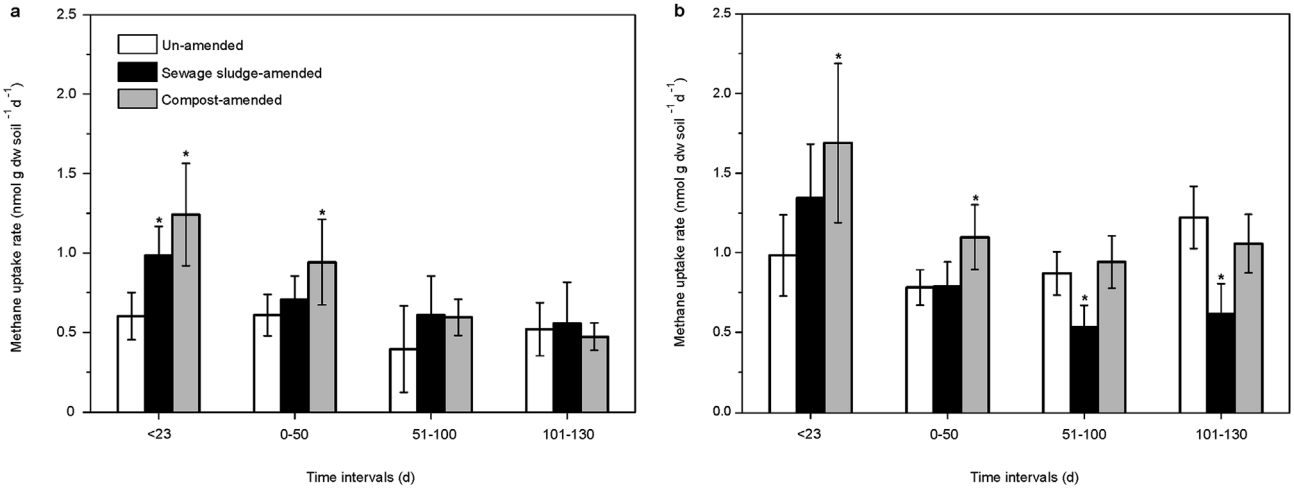

Fig. 1. Methane uptake rates in the residue- and un-amended sandy loam (a) and clay (b) soils showing that residue amendments transiently stimulated soil methane uptake. The values are given as mean and standard deviation of six independent replicates; three replicates each for the un-labelled- and $^{13}C-CH_4$ incubations. Headspace gas was replenished at days 50 and 100. The level of significance is indicated by an asterisk after pairwise comparison of the residue- and un-amended soils per time (*t*-test; $P < 0.05$).

3.2. Response of the active and total microbial community to bio-based residue amendments

The ^{13}C-enriched PLFA was extracted to determine the microorganisms assimilating the ^{13}C, hence directly linking methane oxidation to the metabolically active methanotrophs (Bodelier et al., 2009, 2013). The incorporation of ^{13}C into the PLFAs was appreciably higher in the clay soil ranging from 1000 to 9000 pg g dw soil^{-1} than in the sandy loam soil ranging from 200 to 300 pg g dw soil^{-1} (Fig. 3). The ^{13}C-enriched PLFAs were compared to PLFA profiles of cultured methanotrophs to identify the active members of the community (Fig. 4). The PLFA profiles of all cultured methanotrophs used as reference in this study are provided in the Supporting Materials (Table S1). The MDS with the minimum spanning tree analysis show the placement of the ^{13}C-enriched PLFAs relative to PLFAs of the cultured methanotrophs with the shortest distance (i.e., similarity) connecting all the PLFA profiles (Fig. 4). The branched C17 (iC17:0) PLFA, anticipated to be indicative of the as-yet-uncultured high-affinity methanotrophs (Bull et al., 2000) were not detected among the labelled PLFA profiles, indicating that these methanotrophs were not metabolically active (Fig. 3). Instead, the ^{13}C-enriched PLFAs were overwhelmingly comprised of canonical methanotrophs closely related to the genus *Methyloferulla* in the sandy loam soil, and to *Methylosinus trichosporium* species and *Methylocystis* in the clay soil. However, without isolates, we cannot completely exclude that some of these methanotrophs may belong to novel lineages. Apart from the sewage sludge-amended clay soil, the PLFAs from both soils clustered closely together, regardless of residue amendments, showing marginal effect of bio-based residue addition on the active methanotrophic population particularly in the sandy loam soil. Interestingly, the active methanotrophs in the clay soil after sewage sludge amendment was related to canonical gammaproteobacterial methanotrophs; with only one replication showing this result, further studies are needed to substantiate this finding. In addition, the *pmoA*-based qPCR analysis showed that the methanotroph abundance remained largely unchanged after the incubation, and was comparable between amendments within soil types (Figure S1).

MiSeq amplicon sequencing and analysis of the 16S rRNA gene was performed to determine the response of the total bacterial community to residue amendments. Although a compositional shift among the active methanotrophs was not detected following residue amendments, the bacterial community composition changed after sewage sludge addition (Figure S2). The PCA revealed a clear separation of the bacterial community in the un- and sewage sludge-amended incubations in both soils along axis PC2 (Figure S2). Members of the bacterial community related to *Xanthomonadaceae* and *Streptomycetaceae* appeared to be favored after sewage sludge amendment in the sandy loam and clay soils, respectively. The bacterial communities in both the compost-amended soils clustered with the un-amended soils, indicating that compost addition exerted only a marginal effect on the soil bacterial population (Figure S2). Therefore, the total bacterial community was more responsive than the active methanotrophs to residue amendments, and the response was dependent on the type of residue.

Additionally, MiSeq sequencing of the *pmoA* gene was performed to exclude the presence of the as-yet-uncultured high-affinity methanotrophs in the soils. Sequences related to these methanotrophs were not detected in the 16S rRNA gene sequence analysis, and was only detected at a minor overall fraction (< 0.007% of total sequence reads) in the *pmoA* gene.

4. Discussion

4.1. Stimulation of methane uptake in upland agricultural soils

The agricultural soils showed potential for high-affinity methane oxidation as was evident from detectable methane uptake in both the residue- and un-amended soils when incubated under circum-atmospheric methane concentrations (6–40 ppm). The methane oxidation rate was significantly stimulated after residue amendment, but the stimulatory effect was transient (up to 50 days; Fig. 1). This finding was consistent with our previous work (Ho et al., 2015a) despite soils and residues of the previous and current experiments were sampled and processed with a two-year time interval, demonstrating the reproducibility of the methane sink activity and persistence over time. Complete (dissimilatory) methane oxidation was further demonstrated by analysis of the $^{13}C/^{12}C$ isotope ratio of CO_2, which generally showed a significant increase in the residue- and un-amended soils incubated under $^{13}C-CH_4$; the $^{13}C/^{12}C-CO_2$ increase was most pronounced in the clay soil (Fig. 2). Although statistically significant, $^{13}C-CO_2$ was not appreciably enriched after sewage sludge amendment. Nevertheless, methane was consumed (Figs. 1 and 2), suggesting a relatively higher rate of assimilatory methane oxidation (e.g., substrate incorporation as storage polymers; Pieja et al., 2011). Alternatively, the sewage sludge may have stimulated autotrophic growth which reduces the CH_4-derived CO_2 (secondary labelling), but this was not evident in the ^{13}C-enriched PLFA analysis (see discussion Section 4.2; Figs. 3 and 4). Nevertheless, we cannot completely exclude that some

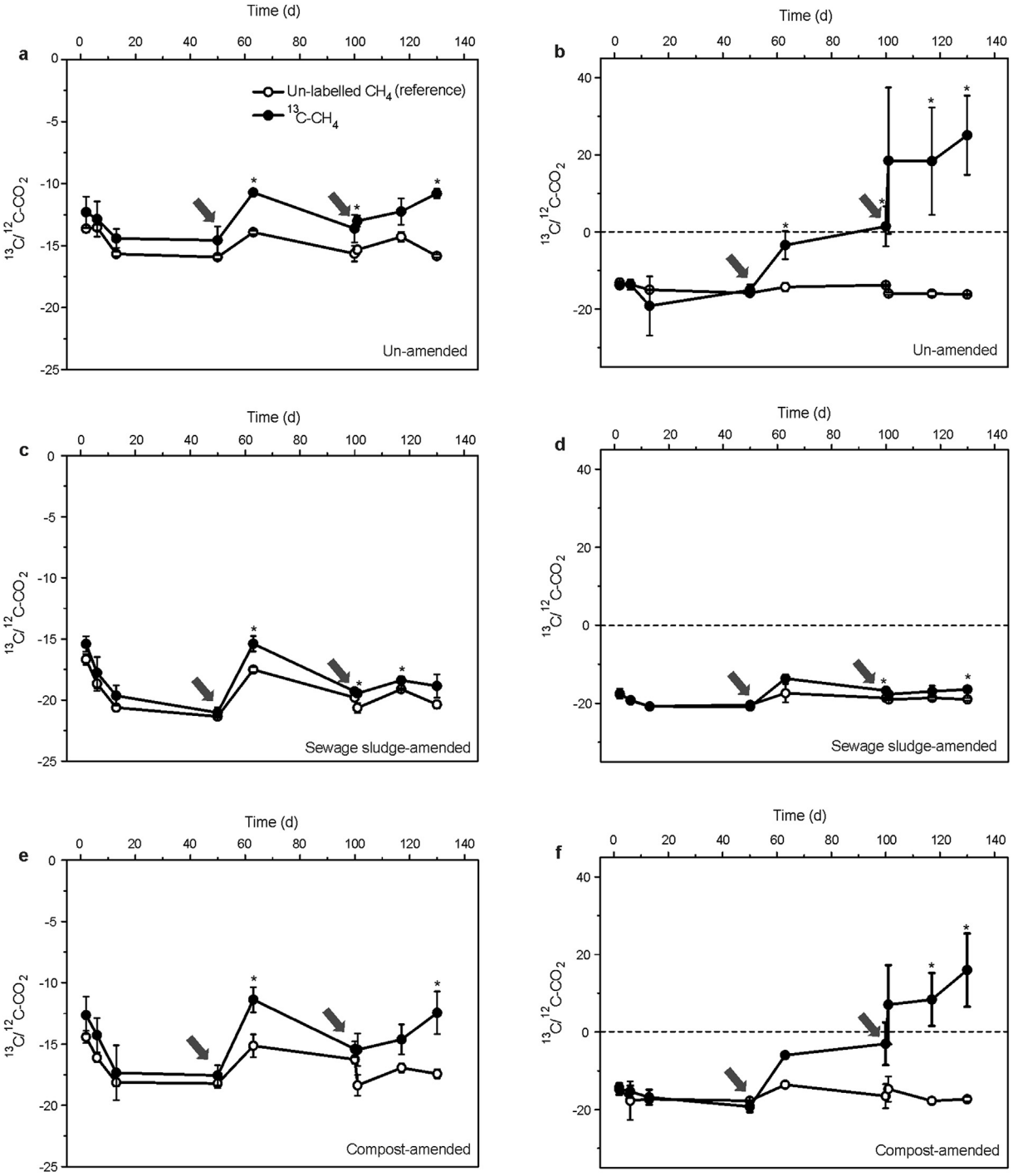

Fig. 2. The $^{13}C/^{12}C{-}CO_2$ ratio showing enrichment of $^{13}C{-}CO_2$ during incubation with un-labelled- and $^{13}C{-}CH_4$. Points are the mean and standard deviation of three independent replicate incubations of the sandy loam (a,c,e) and clay (b,d,f) soils, un-amended (a,b) and amended with sewage sludge (c,d) and compost (e,f). The arrow indicates the time when headspace gas was replenished (days 50 and 100). The level of significance is indicated by an asterisk after pairwise comparison of the un-labelled- and $^{13}C{-}CH_4$ incubations per time and amendment (*t*-test; $P < 0.01$).

alphaproteobacterial methanotrophs (e.g., *Methylosinus*) may have incorporated the $^{13}C{-}CO_2$ in addition to the labelled methane (Yang et al., 2013). In the sewage sludge-amended clay soil however, the lower $^{13}C/^{12}C{-}CO_2$ values reflect the reduced methane uptake capacity > 50 days (Fig. 1). Regardless, it is clear that $^{13}C{-}CH_4$ was sufficiently incorporated into the PLFA to enable detection of the active methanotrophs (Figs. 3 and 4). Hence, with relatively low or no methane production/emission (Ho et al., 2015a,b), the two upland agricultural soils appear to be sinks for methane.

Methanotrophs inhabiting the sandy loam and clay soils exhibited distinct apparent cell-specific activity irrespective of the amendments, suggesting different physiological traits inherent to the community members in both soils (Table S2). The cell-specific activity of the clay soil was two orders of magnitude higher ($100–225 \times 10^{-18}$ mol h^{-1} $cell^{-1}$) than the values derived for the sandy loam soil ($4–7.5 \times 10^{-18}$ mol h^{-1} $cell^{-1}$). Assuming an estimated maintenance energy requirement of 2–3 kJ h^{-1} C-mol $biomass^{-1}$ at the incubation temperature (15 °C), a cell-specific activity of $18–27 \times 10^{-18}$ mol h^{-1} $cell^{-1}$ is required to maintain the methanotrophic biomass (Tijhuis et al., 1993; Kolb et al., 2005). As the

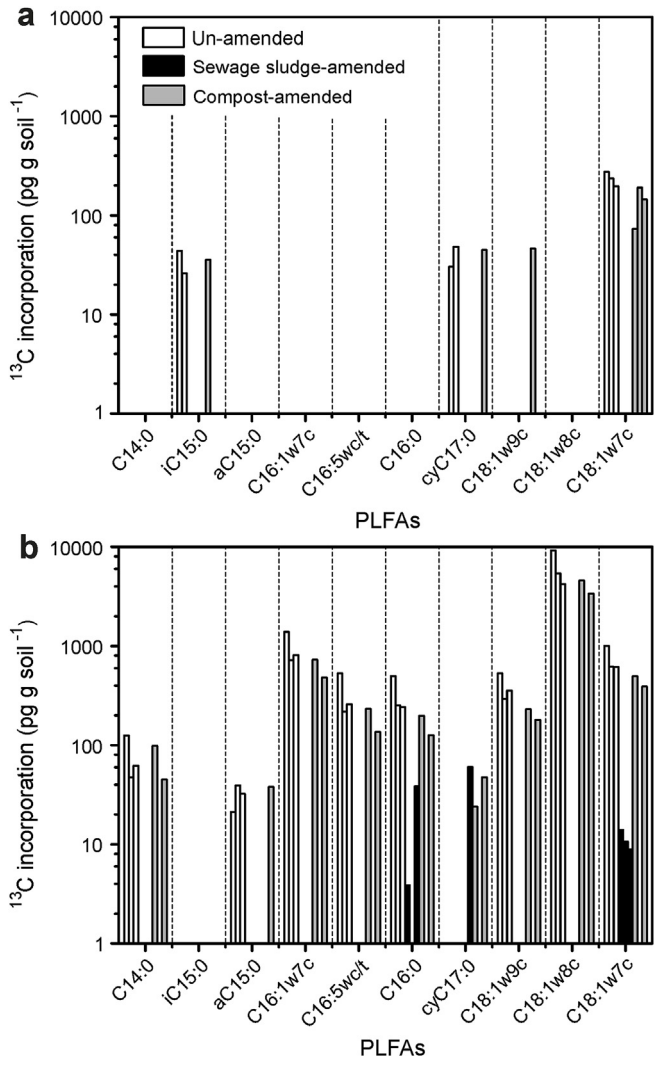

Fig. 3. Incorporation of $^{13}C-CH_4$ into individual PLFA. All replicates are given for incubations of the un-amended (white bars), sewage sludge-amended (black bars), and compost-amended (grey bars) sandy loam (a) and clay (b) soils.

calculated cell-specific activity is 2–7 folds lower than the maintenance energy requirement in the sandy loam soil, we did not anticipate growth of the methanotrophs on (circum-)atmospheric methane alone. This suggests that alternative energy sources (discussed below) are needed to support these methanotrophs *in-situ*.

Recently, it was suggested that canonical methanotrophs may transiently (< 14 days) consume atmospheric methane using accumulated intracellular polymers as reducing equivalents after elevated methane availability in a rice paddy soil (Cai et al., 2016). The capacity to consume atmospheric methane was thus dependent on fluctuations with periods of high methane supply. While the flooding-drainage regime leading to periods of methane production punctuated with times of only atmospheric methane availability is characteristic for low-land rice agriculture (Krüger et al., 2001), the upland agriculture soils do not experience such regime, yet (circum-)atmospheric methane consumption was sustained in these soils (Fig. 1). Contrastingly, the higher cell-specific activity in the clay soil suggests that the methanotrophs may rely on atmospheric methane for maintenance. Changes in *pmoA* gene copies during the incubation was not appreciable, indicating marginal cell replication over 130 days (Figure S1). Therefore, we do not anticipate major compositional shifts in the methanotrophic community during incubation.

4.2. Canonical alphaproteobacterial methanotrophs as a methane sink in upland agricultural soils

Unexpectedly, the active microorganisms almost exclusively belonged to canonical alphaproteobacterial methanotrophs, as resolved by the ^{13}C-enriched PLFA profiles determined after incubation. ^{13}C incorporation into the predominant PLFAs in the clay soil (1000–9000 pg g dw soil^{-1}) was appreciably higher than in the sandy loam soil (200–300 pg g dw soil^{-1}), consistent with the higher cell-specific activity (Fig. 3). The profiles in both soils corresponded to different members of the alphaproteobacterial methanotrophs (Fig. 4). ^{13}C was incorporated into PLFAs that were representatives of the genus *Methyloferulla* in the sandy loam soil, and to *Methylosinus trichosporium* species and *Methylocystis* in the clay soil (un- and compost-amended), showing divergent communities in both soils; differences in the community composition between amendments within soil type was less evident (Fig. 4).

Although other microorganisms, for example, nitrifiers may co-oxidize methane as a non-primary carbon source (Jiang and Bakken, 1999) and secondary labelling may have occurred (Neufeld et al., 2007), the ^{13}C-enriched PLFAs were overwhelmingly assigned to cultured methanotrophs under stringent conditions. This indicates that non-methanotrophic methane oxidation and cross-feeding were marginal. The as-yet-uncultured high-affinity methanotrophs were virtually absent (< 0.007% of total sequencing reads) in the *pmoA* gene sequence analysis. Although residue amendments appeared to have a minimal effect on the active methanotrophic community, a clear shift in the general bacterial composition was detected particularly after sewage sludge addition (Figure S2), consistent with previous findings (Ho et al., 2017a). Interestingly, with the exception of *Methylosinus*, the active methanotrophs possess a proven facultative lifestyle, assimilating methane as well as other multi-carbon compounds (e.g., acetate and ethanol; Dedysh, 2011). This may exclude their sole reliance on atmospheric methane for growth. Reducing power derived from other substrates may sustain atmospheric methane oxidation (Pratscher et al., 2011; Cai et al., 2016). Despite *Methylocystis* and *Methylosinus* harbor the *pmoA2* gene which enables methane oxidation at (circum-)atmospheric concentrations (Baani and Liesack, 2008), the methane sink function in upland soils have so far been attributed to the actions of the putative high-affinity methanotrophs (e.g., Knief et al., 2003; Kolb et al., 2005, 2009). Here, we show that native soils can potentially retain their methane sink function after conversion to agriculture, and that in contrast to native soils, (circum-) atmospheric methane oxidation in agriculture-impacted soils is facilitated by the canonical alphaproteobacterial methanotrophs.

Placing our findings alongside previous studies (Knief et al., 2003; Maxfield et al., 2008; Bodelier et al., 2009; Cai et al., 2016) and extrapolating to a broader context, we predict that distinct populations of active high-affinity methanotrophs predominate in native and anthropogenic-impacted (including agricultural lands) upland soils. Because of their relatively low abundances likely constrained by atmospheric methane supply, and vulnerability to perturbation, the as-yet-uncultured high-affinity methanotrophs thrive in native environments (Knief et al., 2003; Kolb et al., 2005; Maxfield et al., 2008; Levine et al., 2011; Malghani et al., 2016). Conversely, canonical methanotrophs capable of oxidizing (circum-)atmospheric methane, may also utilize methane at higher concentrations upon availability, and are remarkably resilient to environmental stress (Cai et al., 2016; Ho et al., 2016; 2017b). These methanotrophs thrive in anthropogenic-impacted environments, which imply that canonical methanotrophs are robust methane sinks in agriculture-impacted landscapes. Therefore, we provide evidence for an overlooked role of canonical methanotrophs as methane sinks in upland soils, expanding the inventory of (circum-) atmospheric methane-oxidizers in this environment.

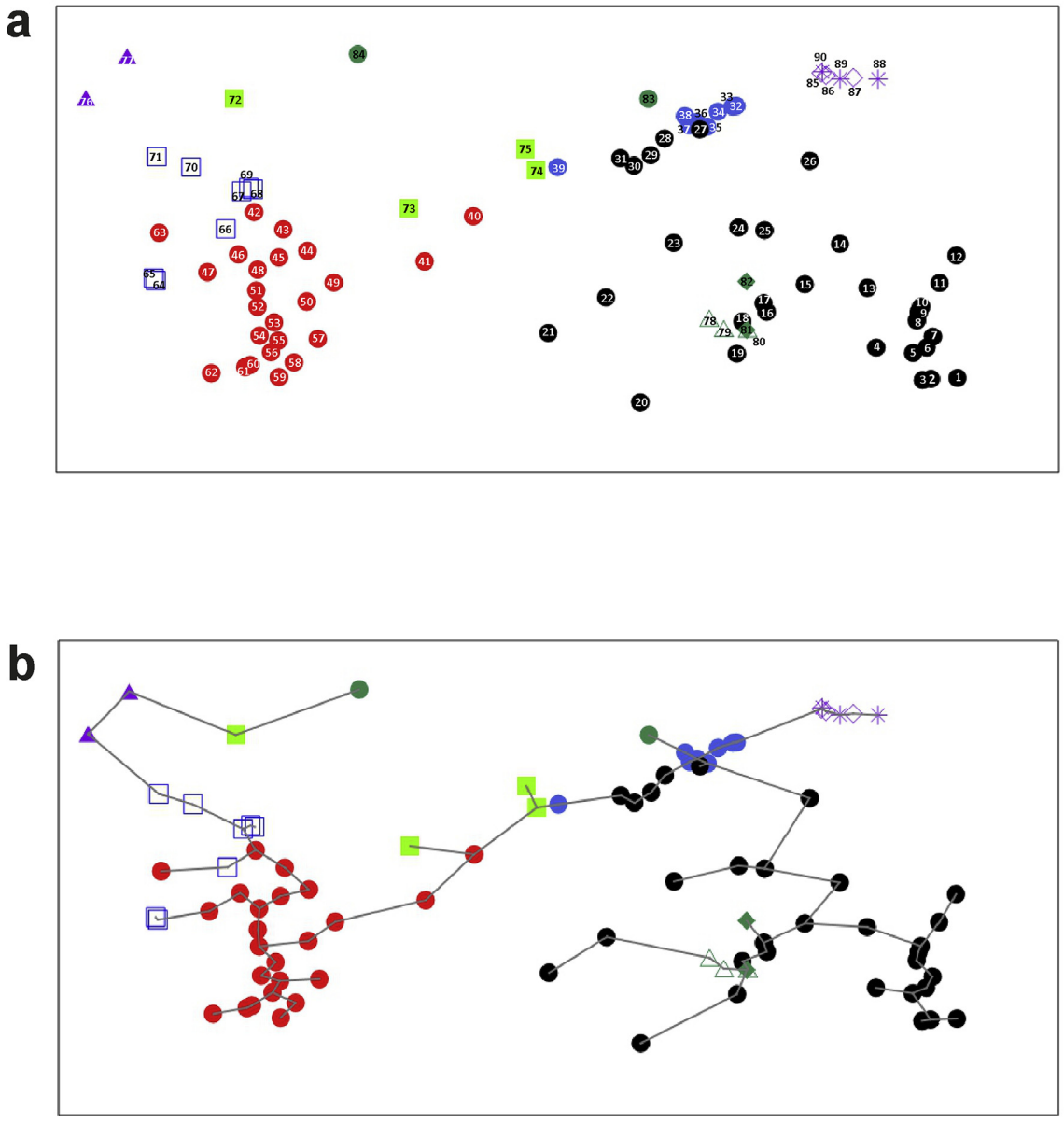

Fig. 4. The active methanotrophic community composition based on the ^{13}C-enriched PLFA profile. Multi-scale dimensional scaling (MDS) of PLFA profiles of methanotroph cultures (expressed as percentage of total PLFA content) and ^{13}C-enriched profiles of soil samples (expressed as percentage of ^{13}C incorporated in separate PLFAs of the total PLFA ^{13}C uptake) (a). The two-dimensional distances between samples in the MDS show the relative similarity between samples. In (b), the identical MDS plot is given, but with a minimum spanning tree analysis displaying the shortest distance (i.e., similarity) to connect all PLFA profiles, resulting in relating to the nearest neighbor for every sample. Green open triangles: un-amended clay soil (78–80); Green diamonds: compost-amended clay soil (81–82); Green dots: sewage sludge-amended clay soil (83–84). Open purple squares: un-amended sandy loam soil (85–87); Purple stars: compost-amended sandy loam soil (88–90). ^{13}C-enriched PLFA was not detected in the sewage sludge-amended sandy loam soil. Black dotes (1–31) represent alphaproteobacterial methanotrophs (*Methylocystis,* and *Methylosinus* genera). Royal blue dots (32–39) represent alphaproteobacterial methanotrophs belonging to the family Beijerinckiaceaea (*Methyloferulla*, *Methylocella*, and *Methylocapsa* genera). Red dots (40–63) represent gammapreoteobacterial methanotrophs within the type Ia subgroup (*Methylomonas*, *Methylomicrobium*, *Methylomarinum*, *Methylobacter*, *Methylosarcina*, *Methyloglobus*, *Methylosphaera, Methyloprofundus*, *Methylovulum,* and *Methylosoma* genera). Open blue squares (64–71) represent gammaproteobacterial methanotrophs within the type Ib subgroup (*Methyloparacoccus*, *Methylomagnum*, *Methylocaldum*, *Methylogae*, and *Methylococcus* genera). Green squares (72–75) represent gammaproteobacterial methanotrophs within the type Ic subgroup (*Methylothermus*, *Methylohalobium*, and *Methylomarinum* genera). Purple triangles (76–77) represent verrucomicrobial methanotrophs (*Methylacidiphylum* genus). Data and literature references associated to the PLFA profiles from cultured methanotrophs can be found in the Supplementary Material (Table S1). The identity of the methanotrophs corresponding to the number code is given in Table S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2018.12.020.

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